Docket No.: 01017/40451C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Brockhaus et al.

Application No.: 08/444,791 Group Art Unit: 1644

Filed: May 19, 1995 Examiner: R. Schwadron, Ph.D.

For: Human TNF Receptor

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. WERNER LESSLAUER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Dr. Werner Lesslauer, do hereby declare and state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced application. I am familiar with the contents of the above-identified U.S. patent application and I am providing this declaration to make available to the Examiner additional data relevant to the invention claimed.

2. Attached hereto as Exhibit A is an affidavit in German that I supplied to the European Patent Office regarding counterpart European patent application no. 99100703.0 (European patent publication no. EP 0 939121 B1).

3. Attached hereto as Exhibit B is an English translation of the affidavit. I hereby confirm my belief in the truth of the statements in this English translation for submission to the U.S. Patent and Trademark Office in the above-identified application.

4. The experiments described in Exhibits A and B compared the activity of a recombinant soluble fragment of the human p75 TNF receptor (p75sTNFR) to the activity of a recombinant immunoglobulin (Ig) fusion protein of p75sTNFR referred to as "p75sTNFR/IgG." The recombinant Ig fusion protein consists of the soluble extracellular domain of the 75 kD TNF receptor, the cloning of which is described in the specification in Example 8, fused to a fragment of the heavy chain of a human IgG3 molecule which is missing the CH1 domain and contains the hinge, CH2 and CH3 domains. This p75 fusion protein is described, *inter alia*, at page 11, lines 1-14.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date December 3, 2004 Werner Lesslauer

Dr. Werner Lesslauer

Eidesstattliche Erklärung

Ich, Werner Lesslauer, Dr. med., Dr. phil. nat., Privatdozent, zur Zeit Gastprofessor der Yale University School of Medicine, Dept. Epidemiology & Public Health and Immunobiology, 60 College Street, New Haven, CT 06520-8034, USA, mache die folgende eidesstattliche Erklärung.

Vom Jahr 1987 bis Ende Juni 1999 war ich in den Biologie-Abteilungen der Zentralen Forschungseinheit und der Forschungsabteilung Zentrales Nervensystem der Hoffmann –La Roche AG in Basel (Schweiz) tätig, zuletzt als wissenschaftlicher Experte verantwortlich für die Leitung verschiedener Forschungsgruppen in den Bereichen der Protein-, Zell- und Molekularbiologie. Seit Anfang September 1999 bin ich als Gastprofessor an der Universität Yale tätig. Meine gegenwärtigen Forschungsprojekte betreffen von pro-inflammatorischen Cytokinen wie zum Beispiel TNFα oder Lymphotoxin (gemeinsam als "TNF" bezeichnet) vermittelte interzelluläre Kommunikation, die von den zellulären Rezeptoren dieser Cytokine aktivierten intrazellulären Signaltransduktions-Mechanismen, und die durch solche Prozesse im Rahmen von Entzündungsphaenomenen ausgelöste organoide Transformation von tertiären lymphoiden Geweben. Im weiteren befasse ich mich mit der Rolle von Cytokin-aktivierter Signaltransduktion in cognitiven Funktionen. Diese Arbeiten stellen somit eine Weiterführung der bei Hoffmann-LaRoche verfolgten wissenschaftlichen Interessen dar. Ein Teil meines Verantwortungsbereichs bei der Hoffmann – La Roche AG umfasste die Entwicklung von Verfahren zur rekombinanten Expression und zum Reinigen und Testen von Proteinen, wie beispielsweise den löslichen TNF – Rezeptoren ("sTNFR") und p75TNF – Rezeptor - Immunoglobulin - Fusionsproteinen ("p75sTNFR/IgG"). Diese Rezeptor-Fusionsproteine wurden durch die Fusion der löslichen extrazellulären Domäne des p75TNF – Rezeptors, p75sTNFR, die selbst TNF bindet, mit einem Fragment der schweren Kette eines humanen IgG – Moleküles, das praktisch dem Fc-Teil entspricht, mit biotechnologischen Verfahren konstruiert.

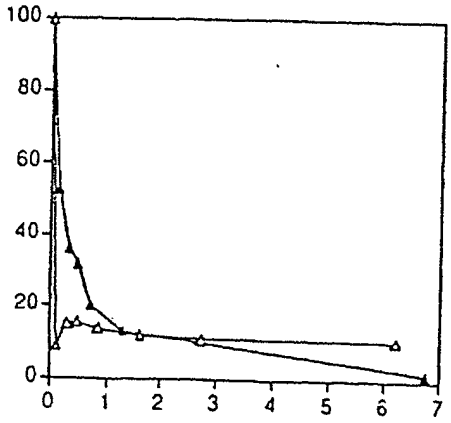
Weiterhin bin ich einer der Miterfinder der vorliegenden Europäischen Patentanmeldung mit der Anmeldenummer 99.100703.0, die solche p75sTNFR/IgG beansprucht. Ich war sowohl an der Erfindung, der Herstellung und dem Testen dieser Fusionsproteine beteiligt.

Gegenstand der vorliegenden eidesstattlichen Erklärung sind die im Vergleich zu der löslichen extracellulären Domäne des p75TNF – Rezeptors p75sTNFR überraschenden Eigenschaften von p75sTNFR/IgG. Zum Zeitpunkt, in dem p75sTNFR/IgG erstmals konstruiert, exprimiert und getestet wurde, gab es Vorstellungen über die räumliche Struktur von TNFα. In dem betreffenden Proteinkristall lag TNFα als Trimer vor und es wurde vermutet, dass dies nicht allein eine Folge der Kristallisierung war, sondern dass das TNFα-Trimer auch die biologisch aktive Form ist. Die räumliche Geometrie der Rezeptor-Bindungsstelle war jedoch unbekannt. Es wäre durchaus möglich gewesen, dass die Fusion mit IgG - Fragmenten ein räumliches Gebilde geschaffen hätte, das wohl TNF-Rezeptorsequenzen enthalten hätte, das aber wegen seiner räumlichen Struktur TNFα überhaupt nicht binden konnte.

Die rasche Elimination und daher kurze Halbwertszeit von p75sTNFR in vivo machte jedoch eine Vergrösserung des Moleküls unerlässlich. Es ist nicht auszuschliessen , dass man sogar eine gewisse Einbusse an Bindungsaktivität in Kauf genommen hätte, um nur eine längere Halbwertszeit und Bioverfügbarkeit zu erreichen. Ueberraschend zeigte das Fusionskonstrukt

jedoch sogar eine sehr gute Bindungsaktivität. Zudem fand sich eine unerwartet höhere kinetische Stabilität, und eine überraschend bessere Inhibierung der Wirkung von TNFα in biologischen Zellkultur-Testen.

Die höhere kinetische Stabilität von p75sTNFR/IgG lässt sich durch den folgenden Versuch I (siehe Figur) veranschaulichen: p75sTNFR/IgG und p75sTNFR werden in separaten Reaktionsgefässen mit radioaktivem TNFα inkubiert, sodass die jeweiligen molekularen Spezies im Komplex mit markiertem TNFα vorliegen. Diese Komplexe werden sodann in neue Lösungen überführt, die einen Ueberschuss an unmarkiertem TNFα enthalten. Wie allgemein in jeder Bindungsstudie wurde auch hier gefunden, dass TNFα mit einer für den jeweiligen Reaktionspartner spezifischen Kinetik an p75sTNFR/IgG und p75sTNFR andockt und wieder dissoziiert. In zeitabhängiger Weise wurde nun die Austauschrate von kaltem TNFα mit dem jeweils an p75sTNFR/IgG und p75sTNFR gebundenen markierten TNFα bestimmt. Eine genaue Beschreibung der experimentellen Technik dieses Versuches wird im Annex gegeben. Die Ergebnisse dieser Experimente sind in der folgenden Figur dargestellt. In dieser ist auf der waagrechten Achse die Zeit in Stunden und auf der senkrechten Achse der Anteil an spezifisch gebundenen radioaktiv markiertem TNFα in % angegeben. Nichtgefüllte Dreiecke stehen für p75sTNFR und gefüllte Dreiecke stehen für p75sTNFR/IgG.



Dieser Figur kann man klar entnehmen, dass am von der Versuchsanordnung her frühest möglichen ersten Messzeitpunkt, d.h. nach etwa sechs Minuten, das gesamte an p75sTNFR gebundene TNFα bereits vollständig ausgetauscht worden war. Hingegen waren bei p75sTNFR/IgG zu diesem Zeitpunkt erst etwa die Hälfte der markierten TNFα Moleküle ausgetauscht worden. Dies bedeutet, dass TNFα mit einer wesentlich langsameren Kinetik von p75TNFR/IgG dissoziiert als von p75sTNFR. Damit wird die Wirkung von TNFα durch

p75sTNFR/IgG wesentlich besser neutralisiert als durch p75sTNFR, da das freigewordene TNFα wieder biologische Aktivität entfalten kann. Diese Eigenschaft lässt das p75sTNFR/IgG Fusionskonstrukt ganz unabhängig von der durch die Vergrösserung des Moleküles bedingten langsameren Elimination in vivo als potenteres pharmakologisches Agens erscheinen.

Diese unvorhersehbare Eigenschaft korreliert auch mit einer unerwartet besseren Inhibierung der Wirkung von TNF durch p75sTNFR/IgG gegenüber p75sTNFR, wie der folgende Versuch II (siehe Tabelle) verdeutlicht. Dabei handelt es sich um einen Versuch in Zellkultur mit weissen, sog. mononukleären Blutzellen, die aus humanem Blut isoliert worden waren. Diese Zellen lassen sich in Kultur durch Behandlung mit mitogenen Substanzen zur Proliferation stimulieren, die dadurch zu Stande kommt, dass einzelne Zellgruppen in der Kultur durch die Mitogen-Behandlung ausgelöst sekundär Wachstumsfaktoren produzieren und in das Kulturmedium abgeben. Der bekannteste dieser Wachstumsfaktoren ist das wohlbekannte Interleukin-2. Daneben hat es sich gerade durch Untersuchungen, die durch die Verfügbarkeit von Reagentien wie p75sTNFR/IgG und p75sTNFR ermöglicht wurden, gezeigt, dass unter anderem auch TNF in den späteren Phasen solcher Kulturen eine zellwachstumsfördernde Aktivität entfaltet. Die Eigenschaft von p75sTNFR/IgG und von p75sTNFR, TNF zu binden und zu neutralisieren, erlaubt nun, diese proliferative Aktivität von TNF zu inhibieren. Das Ergebnis eines derartigen Versuchs ist in der untenstehenden Tabelle festgehalten. In diesem Versuch wurde die Zellproliferation durch den Einbau der radioaktiv markierten Vorstufe Thymidin in die zelluläre DNA gemessen (siehe Annex).

Verwendete Konstrukte	Inhibierung des Einbaus von Deuterium - Thymidin (Tag 7)
p75sTNFR	68 %
p75sTNFR/IgG	86 %

Aus dieser Tabelle ist klar ersichtlich, dass das Fusionsprotein p75sTNFR/IgG gegenüber der löslichen extrazellulären Domäne p75sTNFR eine ueberraschend bessere Neutralisierung der TNF Aktivität , d.h. der Proliferation der Blut- Zellen in Kultur, bewirkt.

Eine derart potentere neutralisierende Wirkung ist in pathologischen Zuständen, die durch zu starke TNFα Freisetzung mitverursacht werden, sehr erwünscht. Es ist hier wichtig, daran zu erinnern, dass TNFα zwar in vielen pathologischen Zuständen ein wichtiger Faktor der Wirtsabwehr ist und damit für den Organismus eine wichtige positive Funktion hat. TNFα hat jedoch ein Janus-Gesicht, und entfaltet in anderen Situationen - sei es durch zu starke Expression, Expression am falschen Ort, oder zur falschen Zeit - krankmachende Wirkungen. Da man bereits zum Zeitpunkt der vorliegenden Anmeldung annahm, dass bei einer Reihe von Krankheiten, wie beispielsweise der rheumathoiden Arthritis, TNFα in der Entstehung der Entzündung und in der Gewebs-Zerstörung in den Gelenken eine Rolle als krankmachender

Mediator spielt, sollten Substanzen welche die Wirkung von TNFα inhibieren auch bei der Behandlung solcher Krankheiten als pharmazeutisch wirksame Substanzen einsetzbar sein. Diese Gedankengänge haben später durch die erfolgreiche Einführung eines p75sTNFR-IgG Präparates in die Therapie der rheumatoiden Arthritis ihre volle Bestätigung gefunden.

ANNEX

Versuch I:

Man inkubiert 1.4µg/ml des p75sTNFR/IgG bzw. 0.75 µg/ml des p75sTNFR in 1ml Phosphat- gepufferter Kochsalzlösung ("PBS"enthaltend 1% foetales Kälberserum) mit 25ng/ml ^{125}I-markiertem TNFα, das in seiner Rezeptor-Bindung von unmarkiertem TNFα nicht zu unterscheiden war. Zum Zeitpunkt Null setzt man dann einen 1000-fachen Ueberschuss nichtmarkiertes TNFα dazu und entnimmt zu verschiedenen Zeitpunkten jeweils kleine Proben von 60µl. Diese Proben gibt man in Millipore 0.22µ MC Filtereinheiten die bereits 20µl einer 50%igen Suspension von 'Protein G Sepharose 4 Fast Flow Beads' ("Sepharose-Kugeln")in PBS mit 1% fötalem Kälberserum enthalten. Damit p75sTNFR/IgG bzw. p75sTNFR an die Sepharose Kugel binden können, wurden diese mit einem gegen den TNF-Rezeptor gerichteten Antikörper vorbeschichtet (1mg Antikörper/ ml Sepharose-Kugeln). Nach Inkubation während 4 min. unter Schütteln wurden die Filtrationseinheiten zentrifugiert (13000 rpm, 30 sek.), und damit das ungebundeneTNFα abgetrennt, während das an p75sTNFR-IgG bzw. p75sTNFR gebundene TNFα auf den Sepharose-Kugeln haften blieb. Damit wurde es möglich, die am jeweiligen Zeitpunkt noch am p75sTNFR-IgG und p75sTNFR gebundene Menge von radioaktiv markiertem TNFα zu messen. Nicht-spezifische Bindung wurde in derselben Weise in Abwesenheit von p75sTNFR/IgG bzw. p75sTNFR bestimmt. 100%ige Bindung wurde in Abwesenheit von nichtmarkiertem TNFα bestimmt. Die Versuche wurden bei 25°C durchgeführt.

Versuch II

Mononukleäre Zellen wurden aus frischem venösen Human- Blut von gesunden Spendern mittels eines Ficoll Paque- Dichtegradienten (Pharmacia, Uppsala, Schweden) in einem Zitratpuffer isoliert. Diese weissen Blut - Zellen wurden zweimal mit einer Phosphat-gepufferten Kochsalzlösung gewaschen und bei einer Dichte von 1.0 x 10^6 Zellen/ml in RPMI 1640 Kulturmedium, das mit 10%igem hitzeinaktivierten fötalen Kälberserum, 100 Einheiten/ml Penizillin, 100µg/ml Streptomycin und 2mM Glutamin supplementiert worden war, kultiviert. Für den Proliferationstest wurden die Zellen in Flachbodenmikrotiterplatten (NUNCLON 1-67008: Roskilde, Dänemark) in 100µl Medium kultiviert. Die Zellen wurden, mit Phytohaemagglutinin(Wellcome, Dartford, England) bei zuvor bestimmten optimalen Konzentrationen im Bereich von 0.5 bis 1.5 µg/ml stimuliert. Zum Startzeitpunkt der Kultur (Tag 0) wurde p75sTNFR/IgG bzw p75sTNFR bis zu eine Konzentration von 10µg/ml zugegeben. Das Kulturmedium wurde nach 3, 4 und 6 Tagen aufgefrischt. Die

Zellproliferation wurde nach 7 Tagen gemessen, wobei den Kulturen sechs Stunden vor dem Ernten mit einem LKB Zellemter 1µCi/Kultur Methyl-3H-Thymidin (1mCi/ml Amersham, Buckinhamshire, England) zugesetzt wurde. Die in die Zellen eingebaute Radioaktivität wurde in einem Betaplatten-Flüssig Szintillationszähler (Pharmacia, Uppsala, Schweden) gemessen. Die dargestellten Werte stellen den Mittelwert von drei Kulturen dar.

Bad Homburg, 8. Oktober 2001 W. Lenhauer

Exhibit B

Affidavit

I, Werner Lesslauer M.D., Ph.D., Private Lecturer, presently Visiting Professor at the Yale University School of Medicine, Dept. Epidemiology & Public Health and Immunobiology, 60 College Street, New Haven, CT 06520-8034, USA, hereby file an affidavit in lieu of an oath:

From 1987 to the end of June of 1999, I was working in the Biology Departments of the Central Research Unit and the Research Department, Central Nervous System, of Hoffmann-LaRoche AG in Basel (Switzerland); toward the end of my activity, I worked as scientific expert and was responsible for the management of different research groups in the fields of protein, cell and molecular biology. At the beginning of September 1999, I began my work as a Visiting Professor at Yale University. My current research projects relate to the intercellular communication mediated by pro-inflammatory cytokines, such as TNFα or lymphotoxin (jointly called "TNF"), the intracellular signal transduction mechanisms activated by the cellular receptor of these cytokines, and the organoid transformation of tertiary lymphoid tissue triggered by such processes in the context of inflammatory phenomena. In addition, I am also working on the role of cytokine-activated signal transduction in cognitive functions. Thus, my current research extends the scientific interests I pursued at Hoffmann-LaRoche. As part of my responsibilities at HoffmannLaRoche AG, I worked on the development of methods for the recombinant expression and for the purification and testing of proteins, such as the soluble TNF receptors ("sTNFR") and p75TNF receptor immunoglobulin fusion proteins ("p75sTNFR/IgG"). These receptor fusion proteins were constructed by means of the fusion of the soluble extracellular domain of the p75TNF receptor, p75sTNFR, which itself bind TNF, and a fragment of the heavy chain of a human IgG molecule which practically corresponds to the Fc portion, using biotechnological methods.

I am also one of the co-inventors of the present European Patent Application with the Application Number 99.100703.0 which claims such p75sTNFR / IgGs. I participated both in the invention and in the production and testing of these fusion proteins.

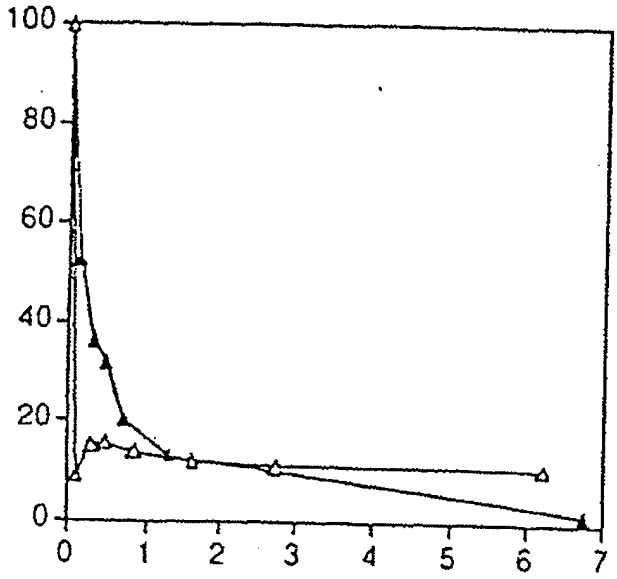
The subject matter of the present affidavit in lieu of an oath concerns the properties of p75s TNFR / IgG which are surprising when comparing them to those of the soluble extracellular domain of the p75TNF receptor, p75sTNFR. At the time when p75sTNFR / IgG was first constructed, expressed, and tested, knowledge of the spatial structure of TNFα was available. In the relevant protein crystal, TNFα was present in the form of a trimer, and it was

hypothesized that this was not only a result of the crystallization but that instead, the TNFα trimer is the biologically active form as well. But the spatial geometry of the receptor binding site was unknown. Thus, it could have been possible that the fusion with IgG fragments created a spatial structure that would have contained TNF receptor sequences but which, due to its spatial structure, was completely unable to bind TNFα.

The rapid elimination and thus the short half-life of p75sTNFR in vivo, however, made it imperative to enlarge the molecule. It cannot be excluded that there might even have been a willingness to accept a certain decrease of the binding activity only to obtain a longer half-life and greater bioavailability. Surprisingly, however, the fusion construct obtained even had an excellent binding activity. In addition, an unexpectedly higher kinetic stability and a surprisingly improved inhibition of the effect of TNF in biological cell culture tests were discovered as well.

The higher kinetic stability of p75sTNFR / IgG can be illustrated on the basis of the following experiment I (see figure): In separate reaction vessels, p75sTNFR / IgG and p75s TNFR are incubated with radioactively labeled TNFα so that the respective molecular species are present in the complex with labeled TNFα. These complexes are subsequently transferred into new solutions which contain an excess of unlabeled TNFα. As is generally the case in any binding study, it was found here as well that TNFα binds to and dissociates itself from p75sTNFR / IgG and p75sTNFR with kinetics specific to the respective reaction participant. Next, the exchange rate of cold TNFα with the labeled TNFα bound to p75sTNFR / IgG and p75sTNFR was determined as a function of time. A detailed description of the experimental technique of this test can be found in the appendix. The results of these experiments are illustrated in the figure below. In this figure, the time in hours is plotted on the horizontal axis, and the percentage of specifically bound radioactively labeled TNFα in % is plotted on the vertical axis. Unfilled triangles stand for p75sTNFR and filled triangles stand for p75sTNFR / IgG.

Exhibit B



This figure indicates very clearly that, based on the experimental set-up, at the earliest possible first time of taking a reading, i.e., after approximately six minutes, the labeled TNFα bound to p75sTNFR had been completely exchanged. For p75sTNFR / IgG, on the other hand, at that point in time, only approximately half of the labeled TNFα molecules had been exchanged. This means that TNFα dissociated with considerably slower kinetics from p75TNFR / IgG than it does from p75sTNFR. Thus, the effect of TNFα is considerably better neutralized by p75sTNFR / IgG than by p75sTNFR, since the liberated TNFα, is able to become biologically active again. This property, quite apart from the elimination in vivo which is slower as a result of the enlargement of the molecule, makes the p75sTNFR / IgG fusion construct a more potent pharmacological agent.

As experiment II below (see table) illustrates, this unforeseeable property also correlates with an unexpectedly superior inhibition of the effect of TNF by p75sTNFR / IgG as compared to p75sTNFR. This test is carried out in a cell culture with white, so-called mononuclear, blood cells which had been isolated from human blood. In culture, these cells can be made to proliferate by treating them with mitogenic substances, which proliferation is propagated by the fact that individual cell groups in the culture produce growth factors which are secondarily triggered by the mitogen treatment and which are released into the culture medium. The best known of these growth factors is the well-known interleukin-2. In addition, tests which were made possible because of the availability of reagents, such as p75sTNFR / IgG and p75sTNFR, had shown that, among other things, TNF also develops a cell growth-promoting activity in the later phases of such cultures. The property of p75sTNFR / IgG and of p75sTNFR to bind and neutralize TNF makes it possible to inhibit this proliferative activity of TNF. The result of such a test is summarized in the table below. In this test, the cell proliferation was measured by

incorporation of the radioactively labeled precursor thymidine into the cellular DNA (see Annex).

Construct Used	Inhibition of the incorporated ^{3}H-Thymidine (day 7)
p75sTNFR	68%
p75sTNFR / IgG	86%

This table illustrates clearly that compared to the soluble extracellular domain p75sTNFR, the fusion protein p75sTNFR / IgG causes a surprisingly superior neutralization of the TNF activity, i.e., the proliferation of the blood cells in culture.

This more highly potent neutralizing effect is very desirable in pathological conditions that are caused by an excessively high TNFα release. In this context, it is important to keep in mind that, although in many pathological conditions TNFα is an important factor of the host's defense and thus plays an important positive role within the organism, TNFα has two faces and, in different situations, develops effects that cause disease -- either by an excessively high expression, or an expression in the wrong site or at the wrong time. Since it was assumed as early as at the time of the present application that in a number of diseases, such as rheumatoid arthritis, TNFα plays a role as a disease-causing mediator in the development of the inflammation and in the destruction of the tissue in the joints, the next step was to assume that it should be possible to use substances that inhibit the effect of TNFα as pharmaceutically effective substances in the treatment of such diseases. Later on, these hypotheses were fully corroborated when a p75sTNFR-Ig/G preparation was successfully introduced into the therapy of rheumatoid arthritis.

Appendix

Experiment I:

1.4 µg / mL of p75TNFR / IgG and 0.75 µg / mL of p75sTNFR were separately incubated in 1 mL of phosphate-buffered physiological saline solution ("PBS" containing 1% fetal calf serum) with 25 ng / mL ^{125}I-labeled, TNFα which, with respect to its receptor binding property, was not distinguishable from unlabeled TNFα. At time zero, a 1000-fold excess of unlabeled TNFα was added, and small samples of 60 µL were taken at different times. These samples were placed into Millipore 0.22 µ MC filter units which already contained 20 µL of a 50% suspension of 'Protein G Sepharose 4 Fast Flow Beads' ("sepharose beads") in PBS with 1% fetal calf serum. To ensure that p75sTNFR / IgG and p75sTNFR can bind to the sepharose beads, these beads had been coated earlier with an antibody directed against the TNF receptor (1 mg of antibody / mL of sepharose beads). After an incubation time of 4 min with shaking, the filtration units were centrifuged (13000 rpm, 30 sec), thus separating the unbound TNFα, while the TNFα bound to p75sTNFR-IgG and p75s TNFR adhered to the sepharose beads. This made it possible to measure the quantity of radioactively labeled TNFα that was still bound to p75sTNFR-IgG and p75sTNFR at a given time. Nonspecific binding was determined in the same manner in the

absence of p75s TNFR / IgG and p75sTNFR. 100% binding was determined in the absence of unlabeled TNFα. The experiments were carried out at 25°C.

Experiment II:

Mononuclear cells were isolated from fresh venous human blood of healthy donors by means of a Ficoll Paque density gradient separator (Pharmacia, Uppsala, Sweden) in a citrate buffer. These white blood cells were washed twice with a phosphate-buffered physiological saline solution and cultured at a density of 1.0×10^6 cells / mL in RPMI 1640 culture medium which had been supplemented with 10% heat-inactivated fetal calf serum, 100 units / mL penicillin, 100 µg / mL streptomycin, and 2 mM glutamine. For the proliferation test, the cells were cultured in flat bottom microtiter plates (NUNCLON 1-67008; Roskilde, Denmark) in 100 µL medium. The cells were stimulated with phytohemagglutinin (Wellcome, Dartford, England) at previously determined optimum concentrations in a range from 0.5 to 1.5 µg / mL. At the time the culture was started (day 0), p75sTNFR / IgG and p75sTNFR, respectively, up to a concentration of 10 µg / mL were added. The culture medium was renewed after 3, 4, and 6 days. The cell proliferation was measured after 7 days; 6 hours prior to harvesting with the LKB cell harvester, 1 µCi of methyl-^{3}H-thymidine (1 mCi / ml, Amersham, Buckinghamshire, England) per culture was added. The radioactivity incorporated into the cells was measured in a betaplate liquid scintillation counter (Pharmacia, Uppsala, Sweden). The values recorded are the mean value of three cultures.

[handwritten:]

New Haven, October 8, 2001 [signature of W. Lesslauerl

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Soluble Tumor Necrosis Factor (TNF) Receptors Are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists

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ABSTRACT. Two forms (monomeric or dimeric) of the extracellular, ligand-binding portion of the human p80 cell-surface receptor for TNF were used to antagonize TNF activity in vitro and in vivo. The dimeric sTNFR:Fc molecule was a more potent inhibitor of TNF than the monomeric sTNFR (50 to 1000×), as assessed in vitro by inhibition of TNF binding or bioactivity and in vivo by protection of mice from an otherwise lethal injection of LPS. Surprisingly, the dimeric sTNFR:Fc construct demonstrated a beneficial effect even when administered 3 h after a lethal LPS injection (i.e., after serum TNF levels had peaked and receded). To study the mechanism by which the soluble TNFR functions in vivo, serum TNF levels were examined in mice given LPS in the presence or absence of soluble receptor. Administration of a mortality-reducing dose of sTNFR:Fc ablated the rise in serum TNF bioactivity that normally occurs in response to LPS. However, TNF bioactivity was revealed in these "TNF-negative" serum samples when the L929 bioassay was modified by inclusion of a mAb that blocks the binding of murine TNF to the human soluble TNFReceptor. These results indicate that the absence of direct cytolytic activity in the L929 assay was caused by neutralization of TNF, rather than to an absence of TNF in the serum. Moreover, administration of either monomeric sTNFR or low doses of dimeric sTNFR:Fc actually resulted in increased serum TNF levels compared to mice given LPS but no soluble receptor. However, these "agonistic" doses of soluble receptor did not lead to increased mortality when an LD_{60} dose of LPS was given. Thus, dimeric sTNFR are effective inhibitors of TNF and under some circumstances function simultaneously as both TNF "carriers" and antagonists of TNF biologic activity. *Journal of Immunology*, 1993, 151: 1548.

TNF is a polypeptide hormone released by activated macrophages and T cells, which mediates a wide range of biologic functions. In addition to its potential role as a regulator of the normal immune response, TNF is also thought to play a major role in systemic toxicity associated with sepsis (1–6). TNF may also be involved in the pathogenesis of AIDS (7–9) as well as a number of autoimmune and inflammatory diseases (10–13). A molecule that specifically inhibits the biologic activities of TNF may thus have considerable therapeutic utility.

Soluble, extracellular, ligand-binding portions of cytokine receptors occur naturally in body fluids and are believed to regulate the biologic activities of cytokines (14–17). The importance of these molecules as cytokine regulators is underscored by the fact that several pox viruses encode proteins with structural and functional homology to the extracellular portions of the receptors for TNF and IL-1 (18–20). Considerable controversy exists concerning the type of regulatory role naturally occurring soluble cytokine receptors might perform. Although it is likely that such molecules will function as cytokine carriers in an operational sense by altering the biodistribution of the cytokine to which they bind, it is not clear whether such an interaction would serve to agonize or antagonize the biologic

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effects of the cytokine (21, 22). However, experiments in which recombinant soluble receptors have been administered in vivo demonstrate their potential to inhibit immune and inflammatory responses, presumably by acting as antagonists of cytokine activity (23, 24).

There are two distinct cell-surface receptors for TNF: the 80 kDa (p80) and the 60 kDa (p60) receptors, both of which bind TNF-α and TNF-β (25, 26). Given the predominantly trimeric nature of TNF (25) and the apparent requirement for cross-linking of cell-surface TNFR for signal transduction (27), it is likely that dimeric soluble receptor constructs should possess a higher affinity for TNF (28) and therefore function as considerably more potent competitive inhibitors than monomeric sTNFR.[2] This prediction has been verified by the results of recent experiments demonstrating superior TNF inhibitory activity of dimeric Fc fusion constructs of p60 in vitro (29). Although soluble forms of both monomeric and dimeric p60 TNFR have been shown to be beneficial in animal models of sepsis, no direct comparison of the in vivo potency of monomeric vs dimeric receptors in sepsis has been reported. In addition, little information is available concerning the mode of action of such inhibitors in vivo.

Monomeric and dimeric (Fc fusion protein) forms of the p80 TNFR were constructed and compared in vitro and in vivo for effects on TNF biologic activity. The results indicate that the sTNFR:Fc, but not the sTNFR, was effective in reducing mortality associated with LPS administration, at least over the concentration range tested. In addition, the sTNFR:Fc molecule can function simultaneously as both a TNF "carrier" and an antagonist of TNF biologic activity and thus inhibit the lethal effects of LPS by acting as a biologic buffer for TNF.

Materials and Methods

Mice

BALB/c female mice 8 to 10 wk old were purchased from Charles River (Wilmington, MA) and were maintained within a specific pathogen-free environment.

Construction and production of p80 sTNFR and sTNFR:Fc

Recombinant sTNFR was expressed in a CHO cell line using the glutamine synthetase selectable and amplifiable marker. For production, cells cultured to confluence in roller bottles were washed with PBS and then cultured in serum-free medium. Purification of the sTNFR from the CHO supernatant was accomplished in a single affinity chromatography step using a mAb, M1, specific for sTNFR.

Recombinant sTNFR:Fc was expressed in CHO cells using the dihydrofolate reductase selectable and amplifiable marker. Suspension cells were centrifuged and resuspended into serum-free medium in a controlled bioreactor. The product was collected after 7 days. The sTNFR:Fc molecule was purified using protein A affinity chromatography followed by an ion-exchange step.

Concentrations of the purified sTNFR and sTNFR:Fc were determined by amino acid analysis. Endotoxin levels were determined to be <5.6 ng endotoxin/mg sTNFR or sTNFR:Fc using the Kinetic-QCL assay (Whittaker Bioproducts, Walkersville, MD) for detection of Gram-negative bacterial endotoxin. Physical characterization included SDS-PAGE, N-terminal sequencing, and immunoreactivity analyses (K. E. Stramler and H. Madani, unpublished observations). A diagrammatic representation of p80 sTNFR and sTNFR:Fc is shown in Figure 1.

Antibodies to soluble TNFR

The generation of mAb to the human p80 sTNFR has been described previously (30). M1 mAb (rat IgG 2b) and M3 (rat IgG) mAb both bind to the human p80 sTNFR but not to mouse TNFR.

Binding inhibition assay

Human rTNF-α was expressed in yeast as a protein composed of the entire coding region of mature TNF fused to an octapeptide at the N terminus, useful in affinity purification. Purified TNF was radioiodinated as described (18) to a sp. act. of 2×10^{15} cpm/mmol, without loss of biologic activity (measured in an L929 cytolysis assay) or receptor-binding activity (see below).

Inhibition assays were carried out as described (31). Briefly, [^{125}I]TNF-α (0.5 nM) was incubated in binding medium (RPMI 1640, 2.5% BSA, 50 mM HEPES buffer, pH 7.4, 0.4% NaN_3) for 2 h at 4°C with serially diluted inhibitors (human sTNFR:Fc, sTNFR monomer, or unlabeled human rTNF-α) and 2×10^6 U937 cells. Duplicate aliquots were subsequently removed, centrifuged through a phthalate oil mixture to separate free and bound ligand, and the radioactivity was measured on a gamma counter. Nonspecific binding values were determined by inclusion of a 200× molar excess of unlabeled TNF and were subtracted from total binding data to yield specific binding values. Data were plotted and results analyzed as described (31).

L929 bioassay for TNF activity

The protocol used to measure the presence of TNF cytolytic activity using L929 cells as targets has been described previously (32, 33). Briefly, 10 μl of mouse serum, mouse

[2] Abbreviations used in this paper: sTNFR, soluble monomeric human p80 TNFR; sTNFR:Fc, recombinant fusion protein composed of soluble dimeric human p80 TNFR linked to the Fc region of human IgG1: CHO, Chinese hamster ovary.

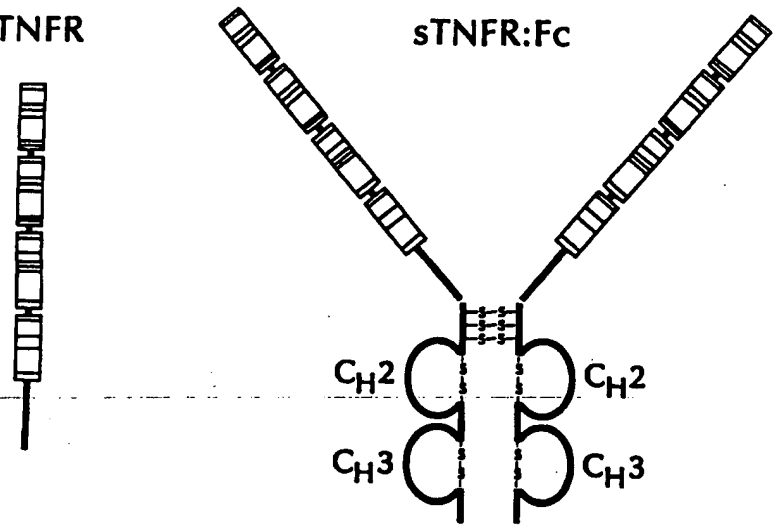


FIGURE 1. Construction of monomeric sTNFR and dimeric sTNFR:Fc molecules. Extracellular portions of the human p80 TNFR cDNA were cloned and produced as described in *Materials and Methods*. In the dimeric sTNFR:Fc molecule three disulfide bonds are depicted. However, the disulfide bond closest to the N terminus is normally used for binding to the Ig L chain and thus, its state (i.e., free cysteine or disulfide bond) in the sTNFR:Fc fusion product is not known.

rTNF-α (Genzyme, Boston, MA), or supernatant from LPS-stimulated RAW 264.7 cells (American Type Culture Collection, Rockville, MD) was serially diluted (50%:50%, v/v) in flat bottom, 96-well microtiter plates. L929 medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) was added to each well, followed by soluble receptors, control proteins, or mAb in a total volume of 30 μl. Ten microliters of actinomycin D was then added (final concentration of 0.1 μg/well; Sigma, St. Louis, MO). Finally, 5×10^4 L929 cells were added to each well (final volume/well = 100 μl) and the plates were incubated at 37°C in 5% CO_2. To prevent the influence of edge effects on the TNF bioassay, only the inner wells of each plate were utilized. All outer wells received 200 μl of L929 medium only. After 16 h of incubation, the culture medium was removed and replaced with 200 μl of 0.5% crystal violet in methanol/water (1/4). The plate was washed with distilled water and air dried at ambient temperature. One hundred microliters of 2% deoxycholic acid (catalog no. D-6750, Sigma) was added to each well to solubilize the crystal violet and the plates were analyzed on an ELISA plate reader at 562 nm. The negative control consisted of L929 cells in the presence of actinomycin-D. Estimates of serum TNF concentrations were obtained by comparing the TNF activity in the experimental serum samples with the activity obtained with the mouse rTNF-α standard.

LPS-induced mortality

LPS, derived from *Escherichia coli* 0127:B8 (catalog no. DF3132-25, VWR, Seattle, WA), was resuspended at 10 mg/ml in sterile saline and stored at −20°C in small aliquots. The LPS was diluted to the proper concentration and sonicated (CU-6 sonicator; Branson, Shelton, CT) for 1 min before injection. BALB/c female mice (18 to 20 g) were injected i.v. with an LD_{60} to LD_{100} dose of LPS (300 to 400 μg) in 0.2 ml of saline. The LPS was injected either alone or in conjunction with sTNFR, sTNFR:Fc or control protein, human IgG (catalog no. I-4506, Sigma). In some experiments, mice were injected with LPS i.v. followed at 2, 3, or 4 h with an i.v. injection of soluble receptor or control protein. Survival was monitored for at least 5 days and, in some experiments, the mice were observed for a maximum of 4 wk. However, no further mortality occurred after the initial 5-day observation period.

Results

In vitro neutralization of TNF activity by soluble TNFR

The ligand binding characteristics of sTNFR monomer and sTNFR:Fc were determined by cell-based inhibition studies using ^{125}I-human rTNF-α and U937 cells expressing surface p80 and p60 TNFR. Results of these experiments are shown in Figure 2*A*. To generate a robust criterion of the relative activity of the sTNFR:Fc, we have analyzed the binding inhibition data with a simple one-site model to yield a single K_i, which reflects that concentration of inhibitor which mediates 50% inhibition of binding of TNF to cell-surface receptors. As predicted from (1) the multivalent interactions that occur between TNF ligands and receptors and (2) previous studies (29), the sTNFR:Fc ($K_i = 1 \times 10^{10}$ M^{-1}) shows ~50-fold higher affinity for the ligand than does the sTNFR monomer ($K_i = 2 \times 10^8$ M^{-1}). Thus, one might suspect that the sTNFR:Fc molecule would be a better antagonist of TNF biologic activity in comparison to the monomeric sTNFR in vitro and in vivo. To address the biologic efficacy of monomeric (sTNFR) and dimeric (sTNFR:Fc) forms of the soluble p80 TNFR, both molecules were analyzed for their ability to neutralize TNF activity in vitro in the L929 bioassay (Fig. 2*B*). Monomeric sTNFR and dimeric sTNFR:Fc inhibited the ac-

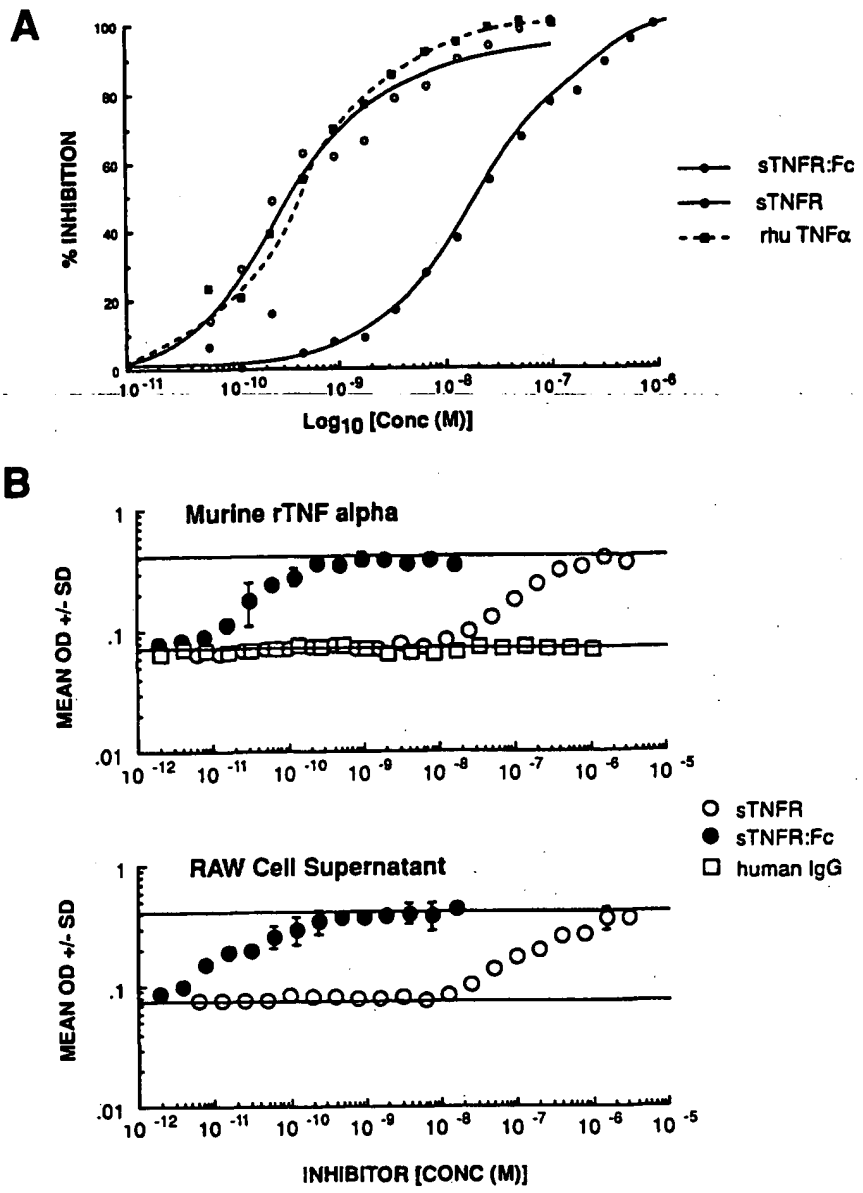


FIGURE 2. Comparison of TNF binding and neutralizing capability of sTNFR and sTNFR:Fc. *A*, U937 cells (2×10^6) were incubated at 4°C for 4 h with 0.5 nM ^{125}I-human rTNF-α in binding medium and varying concentrations of inhibitor (sTNFR:Fc, sTNFR monomer or unlabeled human rTNF-α) in a total volume of 150 μl. Duplicate 70-μl aliquots of the suspension were subsequently removed and microfuged through a phthalate oil mixture to separate free and bound ligand. Radioactivity was measured in a gamma counter and the data were analyzed according to a simple competitive inhibition model. *B*, a constant amount of murine rTNF-α (125 pg/ml) or natural TNF (derived from LPS-stimulated RAW cells, 1/200 dilution) was added to each well of an L929 cytolysis assay in the presence of varying amounts of inhibitors (sTNFR, sTNFR:Fc or human IgG). Details of the L929 cytolysis assay are provided in *Materials and Methods*. The OD of L929 cells in the absence of TNF is indicated by the *upper solid line* (mean OD approximately 0.45) and maximal lysis of L929 cells is indicated by the *lower solid line* (mean OD approximately 0.075).

tivity of mouse TNF (recombinant or natural) in a dose-dependent fashion; however, sTNFR:Fc was approximately 1000-fold more efficient than sTNFR. Identical results were obtained when human rTNF-α was utilized as the ligand (data not shown). Human IgG, used as a control protein, had no effect on TNF activity.

Ability of sTNFR to prevent mortality induced by LPS

We have also compared the biologic efficacy of sTNFR and sTNFR:Fc in vivo in a murine model of LPS-induced septic shock. Various doses of sTNFR:Fc or control protein (human IgG) were mixed with a lethal dose of *E. coli* LPS (400 μg/mouse) and injected i.v. into 18- to 20-g BALB/c female mice. Survival was monitored for 5 days and the results are presented in Figure 3. Treatment of mice with LPS only or LPS and any dose of human IgG resulted in 0 to 10% long term survival. In contrast, 90% of mice treated with LPS plus 100 μg (1.95 nmol) of sTNFR:Fc survived. Beneficial effects of the sTNFR:Fc protein were also evident with doses as low as 10 μg (0.2 nmol)/mouse. In similar studies we have been unable to demonstrate an effect of recombinant monomeric sTNFR on survival even when doses as high as 260 μg (10.35 nmol) were administered (Fig. 4). However, based on the in vitro neutralizing capacity of the monomeric vs dimeric sTNFR (Fig. 2) and the dose of sTNFR:Fc required to effect survival in vivo (Fig. 3), monomeric sTNFR would be predicted to demonstrate efficacy at much higher doses (10 mg/mouse).

The ability of the sTNFR:Fc protein to provide protection when given at various times after LPS administration was also tested. Mice received a lethal dose of LPS (i.v.) followed 2, 3, or 4 h later by sTNFR:Fc (100 μg/mouse). Two to three separate experiments were conducted for each

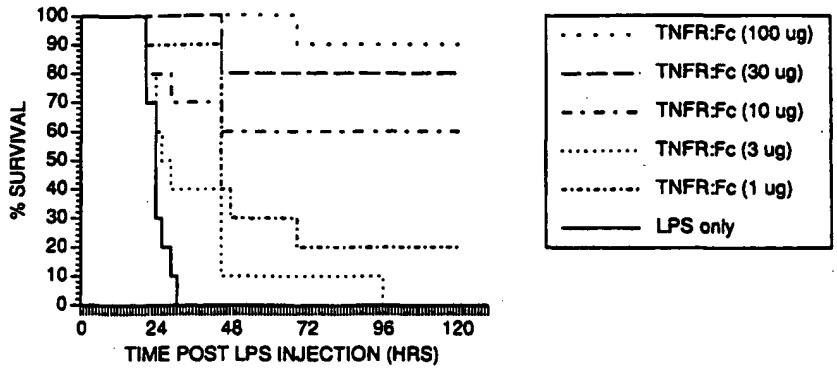
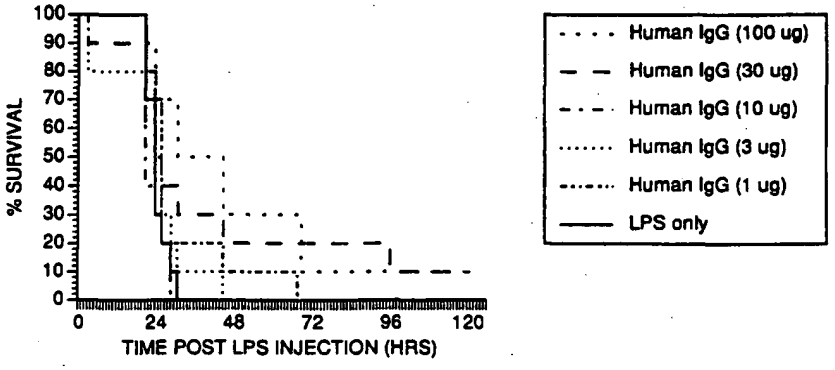


FIGURE 3. Administration of sTNFR:Fc prevents mortality of BALB/c mice injected with a lethal dose of LPS. Various doses of sTNFR:Fc or human IgG, as a control, were mixed with a lethal dose of LPS (400 μg) and injected i.v. into BALB/c mice. Survival was monitored at least once a day for 5 days. In each of three separate experiments, mice treated with sTNFR:Fc at doses of 10 μg or above demonstrated enhanced survival.



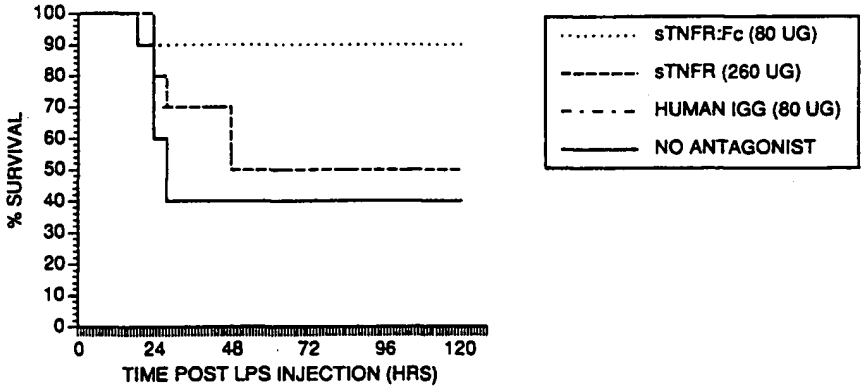


FIGURE 4. Administration of sTNFR does not affect mortality of BALB/c mice injected with a lethal dose of LPS. The procedure was identical to that described in the legend to Figure 3. *Note*: the response of mice treated with human IgG plus LPS overlaps the response of mice treated with sTNFR (260 μg) plus LPS.

time point. All experiments provided similar results and therefore the results were pooled (Fig. 5). The results demonstrate that the administration of sTNFR:Fc was clearly beneficial even when administered up to 3 h after the injection of LPS. In the same experiment, the progression of serum TNF activity after LPS injection was determined in a subset of mice that received LPS only (Fig. 6). These experiments and previous reports (34–36) demonstrate that most of the serum TNF activity was produced during the first 2 h after LPS administration. These results demonstrate that the sTNFR:Fc protein was efficacious even when administered after serum TNF levels had peaked and receded. Thus, the efficacy of the sTNFR:Fc molecule must not be due solely to neutralization of serum TNF bioactivity.

Effect of sTNFR and sTNFR:Fc on serum TNF levels in vivo

To study the mechanism by which sTNFR:Fc protected mice from an otherwise lethal dose of LPS, the effect of the two forms of soluble TNFR on TNF activity present in the serum was examined. Mice were injected with LPS alone (400 μg) or LPS mixed with 100 μg of sTNFR, sTNFR:Fc, or control protein, human IgG. Serum samples were obtained 2 h after injection and assayed for TNF bioactivity (Fig. 7). Mice injected with LPS alone or LPS mixed with human IgG exhibited equivalent amounts of serum TNF activity (approximately 1 ng/ml) 2 h after LPS injection. In contrast, mice treated with LPS plus 100 μg of sTNFR:Fc

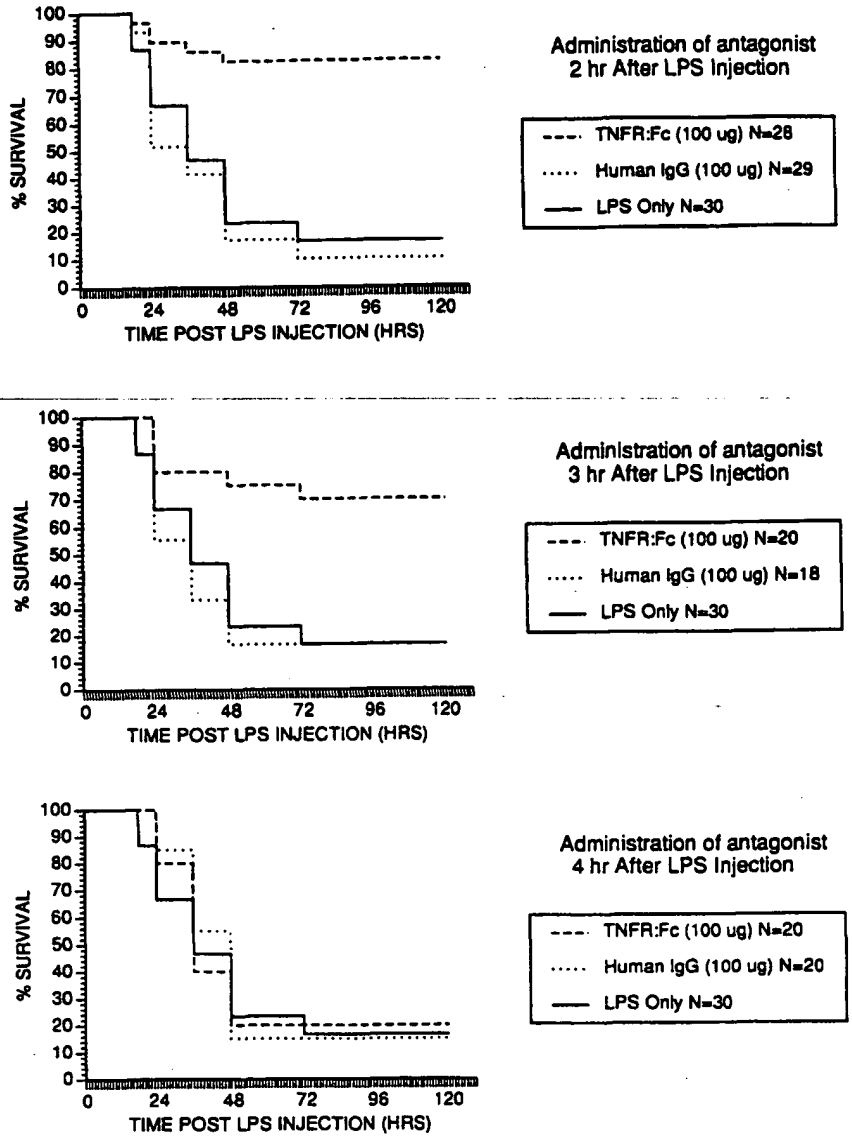


FIGURE 5. Administration of sTNFR:Fc prevents mortality of BALB/c mice even when injected 3 h after a lethal dose of LPS. At 2, 3, or 4 h after i.v. injection of a lethal dose of LPS (400 μg), 100 μg of sTNFR:Fc or human IgG, as a control, were injected i.v. Survival was monitored at least once a day for 5 days. The results represent a compilation of two to three separate experiments.

(which protects mice from the lethal effects of LPS injection, (Fig. 3)) had little or no serum TNF activity as assessed in the L929 assay. Somewhat surprisingly, mice treated with an equivalent dose of monomeric sTNFR (which was not efficacious in survival studies) exhibited serum TNF levels 10-fold higher (10 ng/ml) than control mice treated with LPS only or LPS plus human IgG.

Figure 8 depicts results of an experiment in which the relationship between the dose of sTNFR:Fc and serum TNF activity was examined. Sera obtained from mice injected with LPS alone or LPS plus 1 to 100 μg of human IgG contained detectable TNF activity that titrated in a predictable fashion. Sera obtained from mice 2 h after treatment with 100 or 30 μg of sTNFR:Fc and LPS contained little if any demonstrable TNF activity. Mice injected with 10, 3, or 1 μg of sTNFR:Fc and LPS exhibited serum TNF activity but the sera displayed unusual characteristics. These serum samples demonstrated intermediate levels of TNF activity, which failed to decrease even when diluted to 1/160 (Fig. 8) (data not shown). Because these results were obtained only when mice received LPS and low doses of the sTNFR:Fc, we examined the influence of the sTNFR:Fc on TNF activity in these samples.

Ability of sTNFR:Fc molecules to act as carriers of TNF

Experiments were conducted to determine the effect of blocking the TNF-binding ability of sTNFR:Fc molecules in vitro in the L929 cytolysis assay. To this end, we utilized a mAb (M1) that binds to the sTNFR:Fc molecule and blocks the ability of the soluble human TNFR:Fc protein to bind TNF. Another rat mAb (M3) that binds the sTNFR:Fc molecule but does not block TNF binding was used as a control. To examine the ability of M1 to block TNF binding to sTNFR:Fc proteins, constant amounts of sTNFR:Fc (200

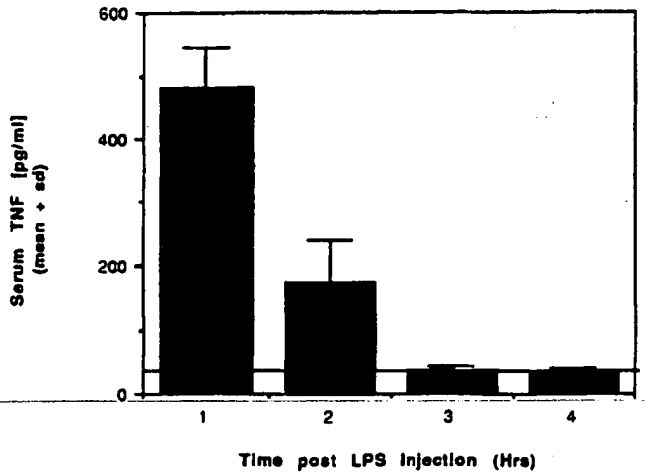


FIGURE 6. Serum TNF levels are elevated for 2 h after lethal LPS injection. Mice were injected with a lethal dose of LPS (400 μg) and serum was obtained at 1, 2, 3, or 4 h. TNF activity was assessed by the L929 cytolysis assay as described in *Materials and Methods.*

ng/ml) and murine rTNFα (125 pg/ml) were added to dilutions of M1, M3, or rat IgG (Fig. 9). The ability of the sTNFR:Fc protein to neutralize the activity of TNF was reversed only in the presence of M1. In addition, full neutralization of the sTNFR:Fc protein (200 ng/ml) required a 10-fold excess (2 μg/ml) of M1.

The effect of addition of M1 to serum obtained from mice 2 h after injection of LPS (400 μg) mixed with 10 or 100 μg of sTNFR:Fc was examined. As previously described (Fig. 8), the serum obtained from mice treated with 10 μg of sTNFR:Fc demonstrated intermediate levels of activity that were not altered by dilution (Fig. 10). Addition of M1 (2 μg/ml) to dilutions of the serum revealed the presence of additional TNF activity, which titrated in a predictable fashion. As expected, addition of control antibody (i.e., M3 or rat IgG) had no effect on the TNF activity. Furthermore, addition of M1, M3, or rat IgG had no effect on serum samples that did not contain the soluble human TNFR:Fc protein (i.e., sera obtained from mice injected with LPS and human IgG), demonstrating that the antibody did not affect the ability of mouse TNF to bind to the indicator L929 cells (Fig. 10). We have also examined serum samples from mice treated with a higher dose of sTNFR:Fc (100 μg) and LPS. In the absence of manipulation these samples did not demonstrate TNF activity in vitro. However, TNF activity was revealed when serum from these mice was treated with M1 but not with M3 or rat IgG (Fig. 10). In fact, maximal activity in the L929 assay of the sera from mice injected with sTNFR:Fc (100 μg) and LPS was still apparent at serum dilutions of 1/100, whereas sera obtained from mice treated with LPS only or LPS plus human IgG demonstrated only small amounts of TNF activity at a dilution of 1/16 (Fig. 10).

To determine whether or not sTNFR:Fc could prolong the presence of serum TNF, mice were injected with LPS and 10 or 100 μg of sTNFR:Fc or human IgG, as described above, and serum samples were obtained at 4 h. The serum samples were assayed in the L929 bioassay in the presence and absence of M1, M3, or rat IgG (Fig. 11). As expected, sera obtained from mice injected 4 h previously with LPS alone or LPS plus human IgG did not contain serum TNF activity. However, sera obtained from mice injected with LPS plus sTNFR:Fc (10 or 100 μg) still contained biologically active TNF, which titrated in a predictable fashion in the presence of M1 mAb. Thus, mice injected with LPS and the soluble human TNFR:Fc protein, even at therapeutic doses, retained increased levels of TNF in the serum that persisted for longer periods of time. However, depending upon the dose of sTNFR:Fc administered, the TNF activity was either (1) enhanced or (2) revealed only upon the addition of a mAb which blocked the binding of TNF to the sTNFR:Fc protein. These observations indicate that the binding of the sTNFR:Fc protein to TNF is reversible and that the inhibition of TNF activity reflects a balance between the presence of sTNFR:Fc, TNF, and endogenous TNFR (either cell surface or soluble).

The carrier function of sTNFR:Fc molecules is not detrimental to the host

As the administration of sTNFR:Fc under some circumstances produced increased levels of serum TNF (Fig. 8) that persisted for at least 4 h (Fig. 11), it was important to determine whether or not the administration of sTNFR:Fc molecules under these circumstances would lead to detrimental consequences. Mice were injected with a dose of LPS (300 μg) which produced intermediate levels of mortality (60 to 70%), such that beneficial or deleterious effects of the TNFR could be observed. Mice treated with sTNFR:Fc at doses ranging from 10 ng to 10 μg demonstrated equivalent or slightly better survival when compared with mice treated with LPS alone or LPS and human IgG (Fig. 12). Further experiments in which lower doses of sTNFR:Fc (100 pg to 1 μg) were utilized yielded similar results (data not shown). Thus, administration of sTNFR:Fc in sublethal models of LPS toxicity had no detrimental consequences on the survival incidence.

Discussion

The data presented in this report demonstrate that a fusion molecule consisting of a soluble form of the extracellular portion of the p80 cell surface TNFR fused to the Fc portion of human IgG1 (sTNFR:Fc) is an effective antagonist of LPS-induced septic shock. An increased incidence of survival in mice given an otherwise lethal dose of LPS was observed when the sTNFR:Fc protein was injected 0 to 3

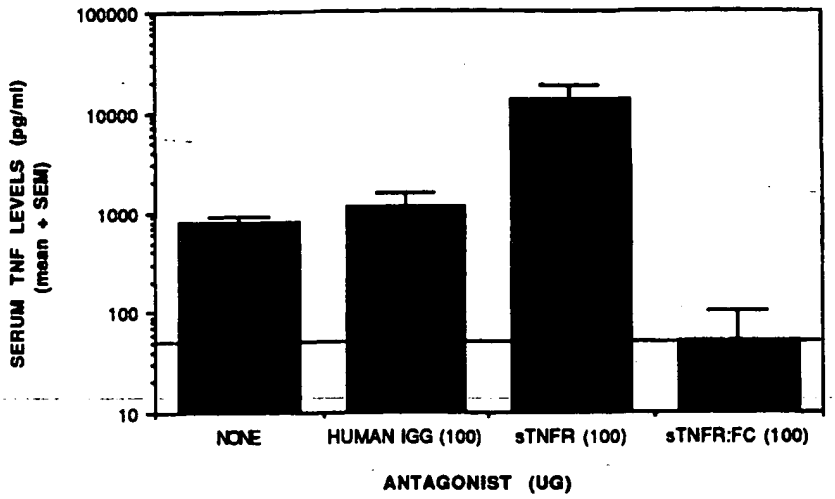


FIGURE 7. The effect of sTNFR vs sTNFR:Fc on serum TNF levels after co-administration of LPS in vivo. LPS (400 μg) was mixed with 100 μg of sTNFR, sTNFR:Fc, or human IgG and administered i.v. to BALB/c mice. Serum samples were obtained 2 h after injection and analyzed for TNF activity in the L929 cytolysis assay. The results were obtained from three to four separate experiments for each treatment group. The sensitivity of the TNF bioassay is approximately 50 pg/ml and is indicated by the *solid line*.

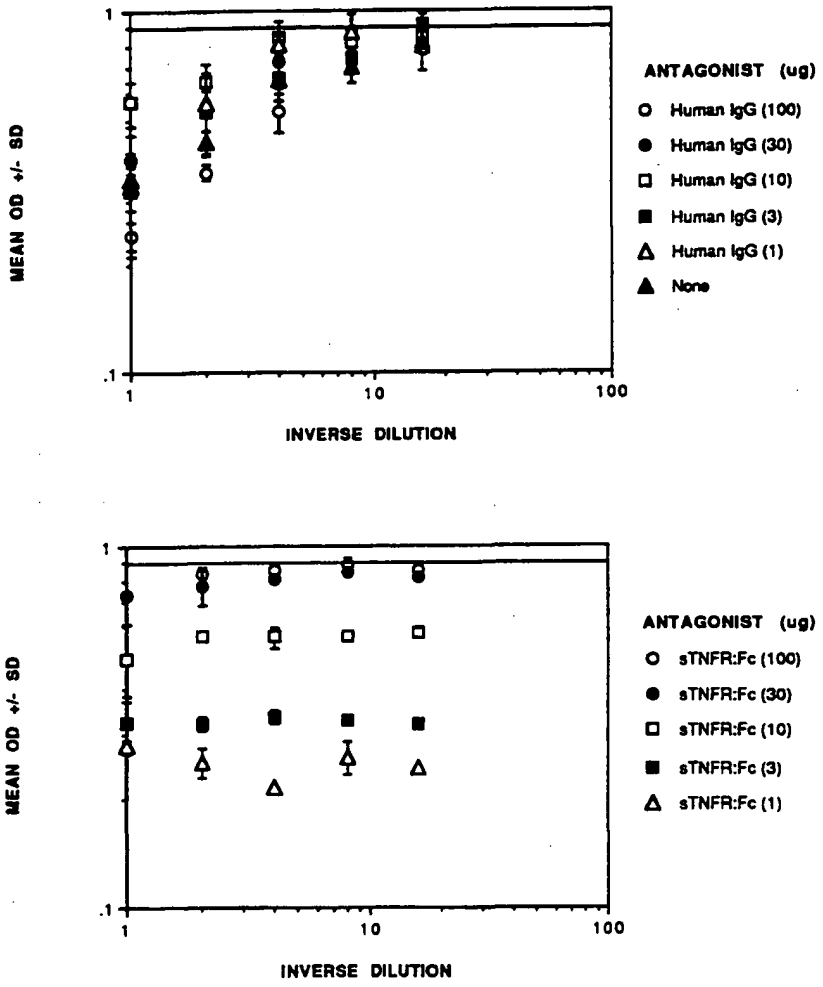


FIGURE 8. Analysis of TNF bioactivity in serum samples obtained 2 h after in vivo co-injection of LPS and sTNFR:Fc. A lethal dose of LPS (400 μg) was mixed with varying doses of sTNFR:Fc or human IgG and injected i.v. into BALB/c mice. Serum was obtained from three mice in each group 2 h after injection. The serum for each group was pooled and analyzed for TNF activity in the L929 assay.

h after LPS administration (Figs. 3 and 5). When administered simultaneously with LPS, doses of sTNFR:Fc as low as 10 μg (0.2 nmol)/mouse were beneficial (Fig. 3). In contrast, administration of up to 260 μg (10.35 nmol) of the monomeric sTNFR failed to affect the incidence of mortality induced by LPS, even when the incidence of mortality in the control group was only 50% (Fig. 4). This difference in efficacy between sTNFR:Fc and sTNFR in vivo may be explained in large part by the higher affinity of TNF for sTNFR:Fc than sTNFR, which results in a substantially greater ability of sTNFR:Fc to neutralize the biologic effects of TNF (Fig. 2). Furthermore, linkage of the sTNFR to the Fc region of Ig imparts a fivefold longer serum $t_{1/2}$ to the sTNFR:Fc molecule after i.v. injection (37), a property

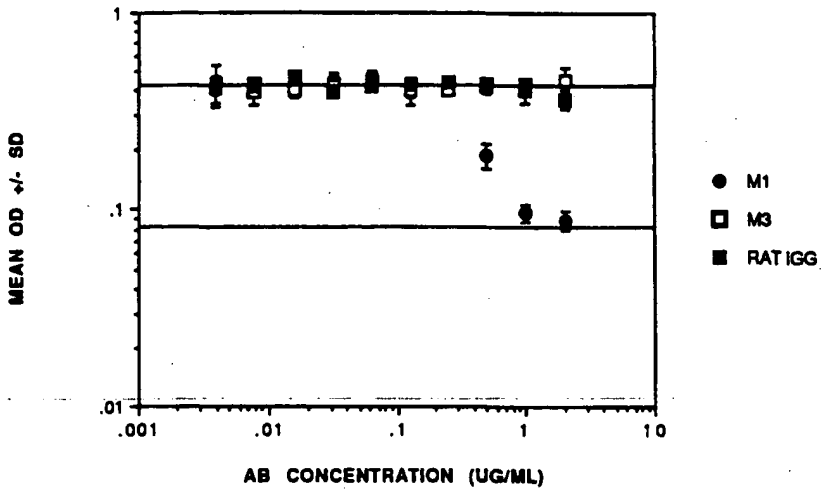


FIGURE 9. Inhibition of the TNF neutralizing capacity of the human p80 sTNFR:Fc molecule by M1 but not M3 mAb. Dilutions of M1, M3, or rat IgG were added to constant amounts of sTNFR:Fc and murine rTNF-α in the L929 assay as described in *Materials and Methods.*

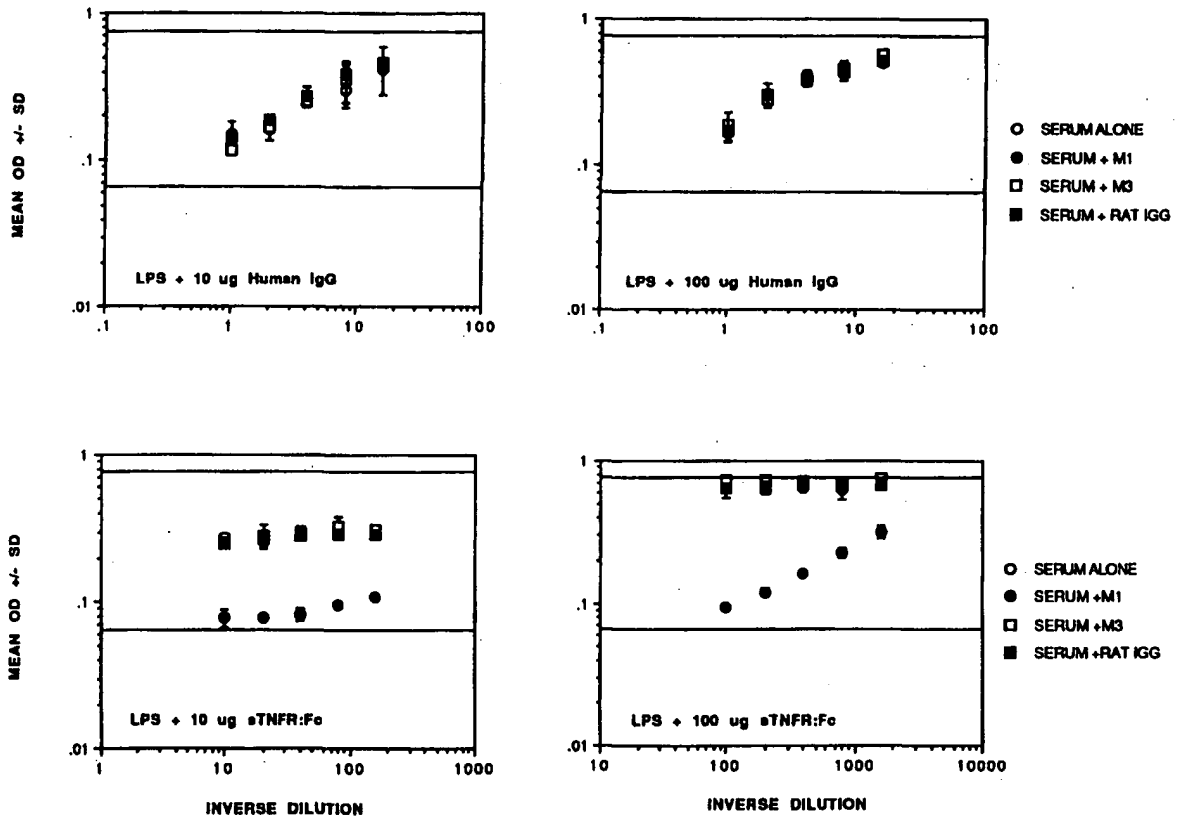


FIGURE 10. Demonstration of serum TNF activity in vitro in the L929 assay in the presence of M1 mAb. Serum was obtained from mice 2 h after injection of LPS (400 μg) mixed with sTNFR:Fc or human IgG. The serum samples were serially diluted and assayed in the L929 cytolysis assay either alone or in the presence of constant amounts (2 μg/ml) of M1, M3, or rat IgG.

that could also influence its efficacy in vivo.

The ability of sTNFR to alter the magnitude and time course of serum TNF after co-administration with LPS in vivo was examined. Sera from mice that received high, life-saving doses of sTNFR:Fc (e.g., 100 μg) failed to exhibit significant levels of TNF bioactivity when assayed directly in the L929 cytolysis assay. However, further experimentation demonstrated that TNF was present in the serum but it was biologically inactive because of the concomitant presence of sTNFR:Fc. TNF activity in these samples was revealed in the presence of a mAb which blocked the ability of the human sTNFR:Fc molecules to bind TNF but did not interfere with the ability of TNF to bind to the murine TNFR on the surface of the L929 indicator cells (Figs. 9 to 11). These results suggest that the sTNFR:Fc protein has a relatively high exchange rate for TNF, such that once TNF is released in vitro, it can be detected if the TNF is inhibited from subsequently binding to free

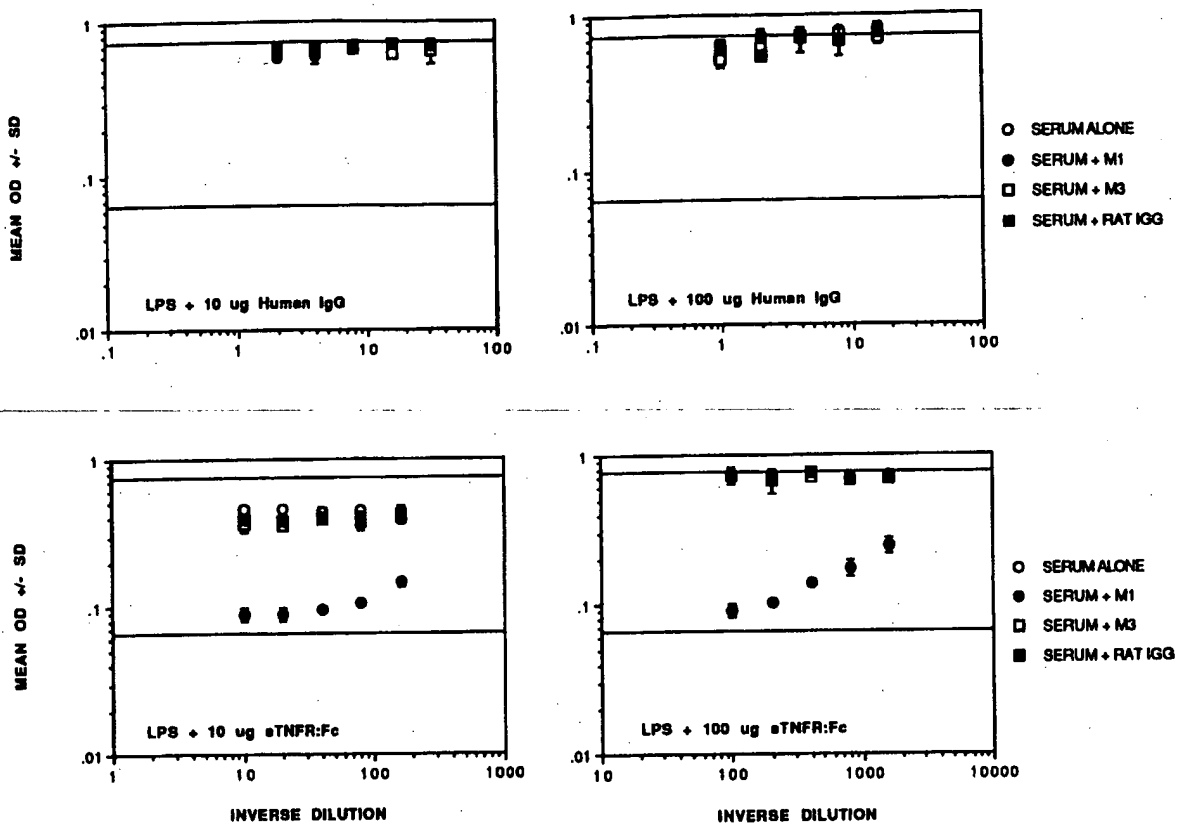


FIGURE 11. Prolongation of serum TNF in vivo by sTNFR:Fc. The protocol was identical to that described in the legend to Figure 10 except that the serum samples were obtained 4 h after LPS injection.

sTNFR:Fc molecules. If TNF is also released from the sTNFR:Fc molecule in vivo, the sTNFR:Fc molecules may function by dissipating the peak in serum TNF levels normally associated with bolus LPS injection.

Soluble TNFR:Fc molecules also function as carriers of TNF in that they alter the rate at which TNF disappears from the serum of LPS-treated mice. Control mice injected with LPS alone or LPS and human IgG had elevated serum TNF levels only during the first 2 h after injection (Figs. 6, 10, 11). However, mice treated with LPS and sTNFR:Fc retained TNF in their serum for at least 4 h (Fig. 11). In support of these data, we have demonstrated that the $t_{1/2}\beta$ of labeled TNF is increased approximately fourfold in vivo when injected concomitantly with sTNFR:Fc (D. Lynch and K. M. Mohler, unpublished observations). These results suggest that the sTNFR:Fc protein functions as an effective antagonist of LPS induced mortality by acting as a biologic buffer for TNF activity.

When mice were exposed to lethal doses of LPS and low doses of sTNFR, which failed to affect mortality incidence, serum TNF levels as detected in the L929 bioassay were elevated in comparison to control mice receiving LPS alone or LPS plus IgG (Figs. 7 and 8). However, despite the fact that low doses of sTNFR increased serum TNF activity, no agonistic activity in terms of mortality could be demonstrated when low doses of sTNFR were administered in conjunction with an LD_{60} dose of LPS (Fig. 12) (data not shown). These data indicate that the agonistic effects on serum TNF activity obtained in vivo in the presence of sTNFR were distinct from the effects of sTNFR on LPS-induced mortality. Alternatively, the sTNFR may function as an agonist only with lethal doses of LPS. If the latter hypothesis is correct, then lower (nonlethal) doses of LPS may induce sufficient quantities of endogenous soluble TNFR so that the administration of exogenous sTNFR:Fc molecules would have relatively minor additional biologic impact.

Several types of TNFR/antibody-based fusion proteins have been described and tested for efficacy in murine LPS-induced mortality models (38, 39). These TNF antagonists include the molecule employed in the present study, composed of the extracellular portion of the p80 cell-surface receptor linked to the Fc region of human IgG1, as well as molecules consisting of fusions between the extracellular portion of the p60 TNFR combined with the Fc region of either human IgG1 (38) or human IgG3 (39). The dose of p60 sTNFR:Fc (4 to 20 μg) (38, 39) and the dose of p80 sTNFR:Fc (10 to 100 μg) (Fig. 3) required to demonstrate efficacy are similar. However, efficacy of the different constructs was influenced substantially by the timing of administration relative to lethal LPS injection. The p80 sTNFR:Fc (human IgG1) construct was efficacious when

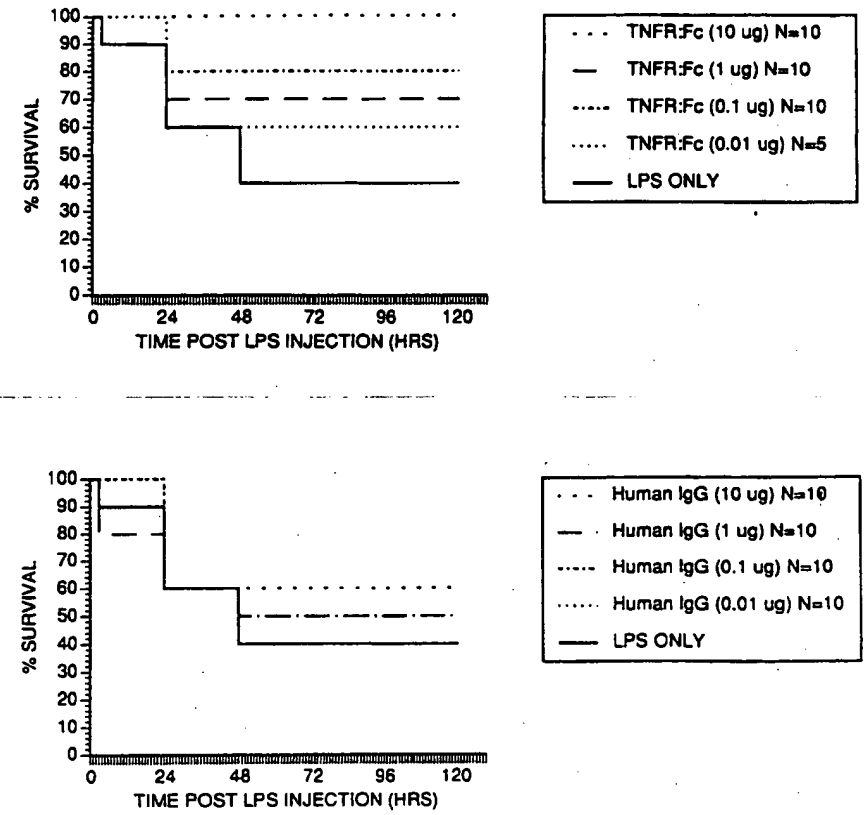


FIGURE 12. Administration of low doses of sTNFR:Fc is not detrimental to the host. BALB/c mice received an LD_{60} dose of LPS (300 μg) premixed with low doses of sTNFR:Fc or human IgG. Survival was monitored at least once a day for 5 days.

administered as late as 3 h after LPS administration (Fig. 5). However, a sTNFR:Fc fusion protein consisting of the p60 sTNFR coupled to human IgG1 was effective only if administered within 1 h after lethal LPS injection (38). In contrast, preliminary reports utilizing the p60 sTNFR coupled with human IgG3 indicated that partial benefits were obtained as late as 3 h after LPS injection (39). Thus, significant differences exist between the published abilities of different sTNFR:Fc fusion proteins to function after LPS administration, and these differences do not appear to correlate with either the particular sTNFR (i.e., p60 or p80) or with the subclass of human IgG utilized for the fusion protein.

The relationship between serum TNF activity and efficacy of the sTNFR:Fc molecule had not been established before the present study. Given the ability of sTNFR:Fc to function effectively when administered as late as 3 h after LPS injection (Fig. 5), it was somewhat surprising to observe that the vast majority of detectable serum TNF activity had already passed by 3 h (Fig. 6). A number of hypotheses, which are not necessarily mutually exclusive, may explain these results. First, the length of time that TNF must be bound to its cell surface receptor prior to the induction of an irreversible biological effect such as cell lysis is unknown. However, studies by Engelberts et al. (40) suggest that TNF must be present for extended periods of time to achieve maximal biologic activity in vitro. Thus, sTNFR:Fc may be able to compete for TNF which has already bound to the cell surface and, in effect, dislodge it before the interaction has occurred for a time sufficient to result in complete biologic signaling. In this regard, the rate of dissociation of radiolabeled TNF from its cell surface receptor in vitro is increased in the presence of either unlabeled TNF (41) or the dimeric sTNFR:Fc (C. Smith, unpublished results). Second, LPS-induced mortality may result from the cumulative effect of TNF. Thus, inhibition of the small amount of TNF present late in the time course might be sufficient to prevent mortality. Third, the therapeutic potential of the sTNFR:Fc molecule may not be related solely to the removal of serum TNF activity. The sTNFR:Fc molecule could function by inhibiting TNF activity in extravascular sites. Finally, LPS-induced toxicity may be mediated at least in part by TNF expressed on the cell surface, which may be masked in the presence of sTNFR:Fc. Regardless of the mechanism of efficacy of the sTNFR:Fc molecule, there is a relatively small window of time, 3 to 4 h after LPS injection, during which serum TNF levels are low and administration of the sTNFR:Fc molecule is still efficacious. These results also suggest that serum TNF levels may not always be a good prognostic indicator for the clinical efficacy of the sTNFR:Fc molecule.

Soluble TNF-binding proteins have been recovered from the urine of normal humans (42, 43) and appear at elevated levels in the serum of cancer patients (44, 45) and in response to endotoxin challenge (46). The biologic role of these TNF-binding proteins is currently under investiga-

tion. Previous investigators have demonstrated that TNF spontaneously loses activity in vitro and, under some circumstances, soluble p60 and p80 TNFR can prevent its spontaneous degradation, thereby enhancing the biological longevity of TNF (47). Our experiments demonstrated that a sTNFR monomer can function as an agonist of serum TNF activity in vivo and a sTNFR:Fc molecule could act either as an agonist or antagonist of serum TNF levels in a dose dependent fashion. Thus, the biologic effect of the soluble TNF-binding proteins isolated from humans will probably vary depending upon the relative concentration of TNF and sTNFR. This concept is supported by recent data of Girardin et al. (48), demonstrating increased concentrations of both TNF and soluble TNFR in the serum of septic patients. In that study, higher ratios of soluble TNFR to TNF correlated with increased probability of survival.

These experiments indicate that the sTNFR:Fc molecule is an effective antagonist of LPS-induced septic shock and are in agreement with a number of studies that have shown the beneficial effects of antagonizing TNF activity in sepsis with either antibody (1–3) or soluble receptors (38, 39). In aggregate these results indicate that TNF plays a central role in mediating the lethality associated with sepsis. However, several lines of evidence suggest that the role of cytokines in sepsis is not yet fully understood. First, antagonism of several cytokines other than TNF (e.g., IFN-γ and IL-1) can also lead to beneficial results (49, 50). Second, anti-TNF antibodies have been reported to have variable therapeutic potential in models of endotoxemia, cecal ligation and puncture and bacterial sepsis (51–53). Further experimentation will be required to determine whether or not the sTNFR:Fc molecule also displays the same spectrum of efficacy. However, the results presented here suggest that the sTNFR:Fc molecule may be a useful therapeutic agent for sepsis and other inflammatory diseases.

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